Supplementary Material

Figure Captions

Supplementary Fig.1 Characterization of DBD-specific DARPins

A Schematic representation of the domain structure of different p63 isoforms and of p53 and p73. TAD: transactivation domain; DBD: DNA-binding domain; TD: tetramerization domain; SAM: sterile α-motif domain; TID: transcriptional inhibitory domain. TA*p63α and GTAp63α contain at their respective N-termini additional sequences that further stabilize the dimeric conformation. ΔNp63α and TAp63γ isoforms form open tetrameric states as they either lack the TAD or the TID which are both necessary to form the closed dimeric state. The oligomeric state is symbolized on the right. The domain definition of used constructs is indicated as numbers corresponding to TAp63α numbering. **B** ITC measurements with the control DARPin and the DBD of p63 showing no binding. The top diagram displays the raw measurement and the bottom diagram shows the integrated heat per titration step**. C** Size exclusion chromatography of either DARPin C14 or G4 with p63 DBD. The elution volume of a sample containing both proteins show a shift to lower volume indicating the formation of a stable complex. A Superdex 75 10/300 column was used. **D** Pulldown experiments with different *in-vitro* translated p63 isoforms and immobilized control DARPin showing no unspecific binding. **E** Fluorescence anisotropy measurements of Alexa 488 labelled control DARPin and purified full-length TAp63α and ΔNp63α isoforms. Size exclusion profiles with indicated peak fractions used for the experiment are shown in **F**. For TAp63α, a Superdex 200 16/600 and for ΔNp63α a Superose 6 16/600 column was used. **G** Detailed analysis of the interface of DARPin C14 with the p63 DBD showing the interacting side chains of the complex crystal structure analyzed by LigPlot[63]. **H** Detailed analysis of the interface of DARPin G4 with the p63 DBD showing the interacting side chains of the complex crystal structure analyzed by LigPlot. **I** Sequence alignments of p63, p53 and p73 DBDs with amino acids highlighted involved in the interaction with DARPin C14 and G4 respectively. Hydrogen bonds are highlighted in red, hydrophobic contacts in blue, unique interactions of DARPin C14 are highlighted with a box and of DARPin G4 with a dashed box. **J** Sequence alignments of DARPin C14 and G4 with highlighted amino acids following the same color code as in (I). Unique interacting amino acids with the p63 DBD are highlighted with a box and numbers referring to numbers shown in (I). **K** Comparison of the DARPin C14-p63 DBD

complex with a complex of the p63 DBD and DNA (PDB code: 3US1). The comparison shows that the DARPin C14 blocks the DNA binding interface of the p63 DBD. **L** Comparison of the DARPin G4-p63 DBD complex with a complex of the p63 DBD and DNA (PDB code: 3US1). The comparison shows that the DARPin G4 blocks the DNA binding interface of the p63 DBD.

Supplementary Fig.2 Characterization of the Tetramerization domain-specific DARPin 8F1

A Structures of the tetramerization domain of p63 (PDB code: 4A9Z) and the oligomerization domain of p53 (PDB code: 1SAF). Each monomer is colored differently. The p63 TD has one additional helix that crosses the tetramerization interface and stabilizes the tetrameric state. This additional helix is missing in p53. **B** ITC measurement with the control DARPin and the TD of p63 showing no binding. The top diagram displays the raw measurement and the bottom diagram shows the integrated heat per titration step. **C** Size exclusion chromatography of DARPin 8F1 with the p63 TD. The elution volume of a sample containing both proteins show a shift to lower volume indicating the formation of a stable complex. A Superdex 75 10/300 column was used. **D** Detailed view of the DARPin 8F1–p63 TD interface showing the most critical amino acid side chains involved in the interaction analyzed by LigPlot[63]. **E** Crucial amino acids are also indicated within the sequences with hydrogen bonds highlighted in red, hydrophobic contacts in blue. **F** Sequence comparison of the p63 TD with the p53 TD as well as with the p73 TD. * indicates identical amino acids, : homologous amino acids and . a lower degree of homology between amino acids. Solid frames indicate residues that are recognized by the DARPin and that are not conserved in the sequences of p53 and p73, frames with broken lines those residues that are identical or homologous.

Supplementary Fig.3 Characterization of the SAM domain-specific DARPin A5

A ITC measurement with the control DARPin and the SAM domain of p63 showing no binding. The top diagram displays the raw measurement and the bottom diagram shows the integrated heat per titration step. **B** Size exclusion chromatography of DARPin A5 with the p63 SAM domain. The elution volume of a sample containing both proteins show a shift to lower volume indicating the formation of a stable complex. A Superdex 75 10/300 column was used. **C** Detailed view of the DARPin A5–p63 SAM domain interface showing the most

critical amino acid side chains analyzed by LigPlot[63]. **D** Crucial amino acids are also indicated within the sequences shown below the structure with hydrogen bonds highlighted in red, hydrophobic contacts in blue. **E** Sequence comparison of the p63 SAM domain with the p73 SAM domain. * indicates identical amino acids, : homologous amino acids and . a lower degree of homology between amino acids. Solid frames indicate residues that are recognized by the DARPin and that are not conserved in the sequences of p73, frames with broken lines those residues that are identical or homologous.

Supplementary Fig.4 Experimental overview of control DARPin experiments and pulldowns with full-length p53 family isoforms

A ITC measurements of the control DARPin with the SAM domains of p63 and p73. Measurements with p53 are not shown, as p53 does not have a SAM domain. **B** ITC measurements of DBDs and **C** TDs of p53, p63 and p73 with control DARPin. **D** Western blot analysis of the pulldown experiments of *in-vitro* translated TAp63α, ΔNp63α, TA*p63α, GTAp63α, TAp63γ as well as p53 and TAp73α and the indicated DARPins. On the left, the level of the different proteins in the lysate is indicated, on the right the pulled down protein levels. All p53 family members contain a myc-tag at their N-terminus that was used for detection with the anti-myc antibody 4A6 (Millipore). The experiment was carried out in biological triplicates and one representative experiment is shown. **E** Bar diagrams of the results shown in the western blots in (D) for all selected DARPins. The data for the p63 isoforms are identical to the data shown in Fig. 1B, 2C and 3C, the results for p53 and TAp73α are added (n=3). **F** Same as (E) but for control DARPin (n=3). **G** Transactivation assay with p53, TAp63γ and TAp73β in H1299 cells. All three family members show high transcriptional activity. The transcriptional activity of all three p53 family members is not significantly influenced by increasing concentrations of co-expressed control DARPin. The bar diagram shows the mean values and error bars the corresponding SD of four technical replicates. Statistical significance was assessed by ordinary one-way ANOVA.

Supplementary Fig.5 Fluorescence labelling of all DARPins.

A All DARPins used in this study were side directed labelled with the fluorophore Alexa 488 via a cysteine introduced at their C-terminus via a glycine-glycine linker. On the left, full chromatograms of runs on an HiTrap Q HP anion exchange column (Cytiva) are shown with

a NaCl gradient from 50 mM to 600 mM. On the right the DARPin peaks of a subsequent run on a size exclusion chromatography Superdex 75 10/300 column (Cytiva) are shown. The 8F1-A488 DARPin seems to interact with the column material and shows therefore a higher elution volume. Absorption traces for excitation with both 280 nm and 493 nm wavelength are indicated. **B** Schematic representation of a DARPin with a cysteine introduced at the C-terminus together with the Alexa Fluor 488 C5 Maleimide before reaction (left). Site-directed fluorophore labelled DARPin at its C-terminal cysteine as used in the fluorescence anisotropy experiments (right).

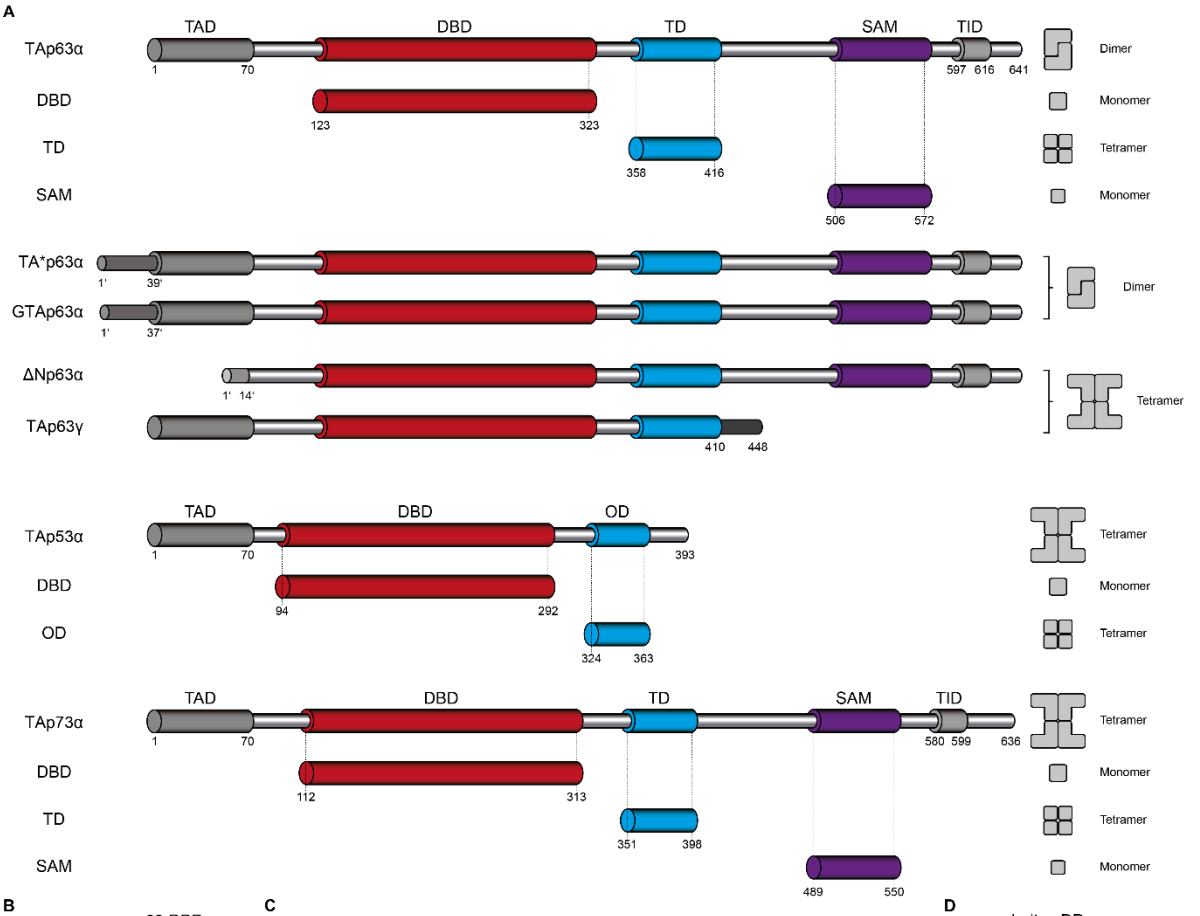
Supplementary Fig.6 DNA pulldown experiments with p53 and TAp73α and cell viability assay

A DNA-pulldown experiments with p53, **B** TAp73α and an immobilized DNA oligomer containing the 20bp binding site of the human p21 promoter. Pre-incubation of the indicated proteins with DARPin C14 or DARPin G4 significantly inhibits interaction with the DNA oligomer while a control DARPin does not significantly prevent binding. Corresponding western blot results are shown above the bar diagram. The relative pulldown efficiency as normalized to no DARPin is shown on the y-axis (n=3). The bar diagram shows the mean values and error bars show the corresponding SD of three biological replicates. Statistical significance was assessed by ordinary one-way ANOVA. **C** Schematic overview of the fusion construct design and cleaving mechanism. DARPins C14, G4 or control DARPin were fused N-terminal to TAp63α with a T2A peptide in between. The T2A peptide is a viral oligopeptide derived from thosea asigna virus which causes the ribosome to skip the formation of a glycyl-prolyl peptide bond due to steric hindrance. This process is called ribosome skipping and causes the cleavage of the translated peptide from its downstream peptide leading to the translation of separated DARPin and TAp63α. **D** Timeline of viability assay over 89h after 0.5 µM or **E** 0.7 µM DOX treatment showing reduction of the number of viable cells of a TAp63α and control DARPin expressing stable cell line compared to cells stably expressing TAp63α and either C14 or G4. All data are normalized to treatment with DMSO only. The standard deviations obtained from biological triplicates are shown.

Supplementary Table 1 Overview of the ITC parameters of the interaction between the selected DARPins and target domains.

Supplementary Table 2 Overview of the fluorescence anisotropy parameters of the interaction between the selected DARPins and target proteins.

Supplementary Table 3 Summary of DARPin-p63 domain complex crystal structure data collection and refinement statistics.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TD
SAM
TID
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Dimer
DBD
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Monomer
TD
358
416
Tetramer
SAM
506
572
Monomer
TA*p63α
1'
39'
Dimer
GTAp63α
1'
37'
ΔNp63α
1'
14'
Tetramer
TAp63γ
410
448
TAp53α
OD
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Tetramer
94
292
Monomer
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Tetramer
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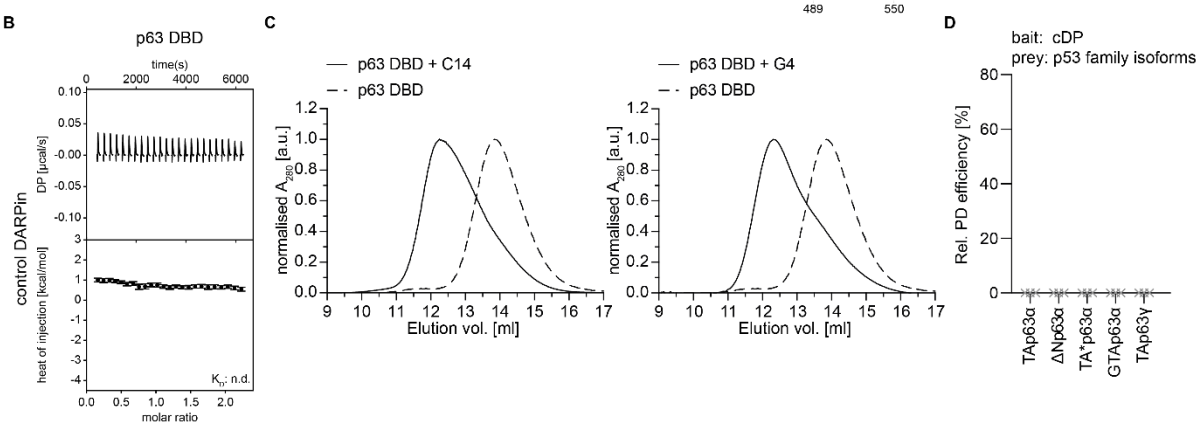
550
Monomer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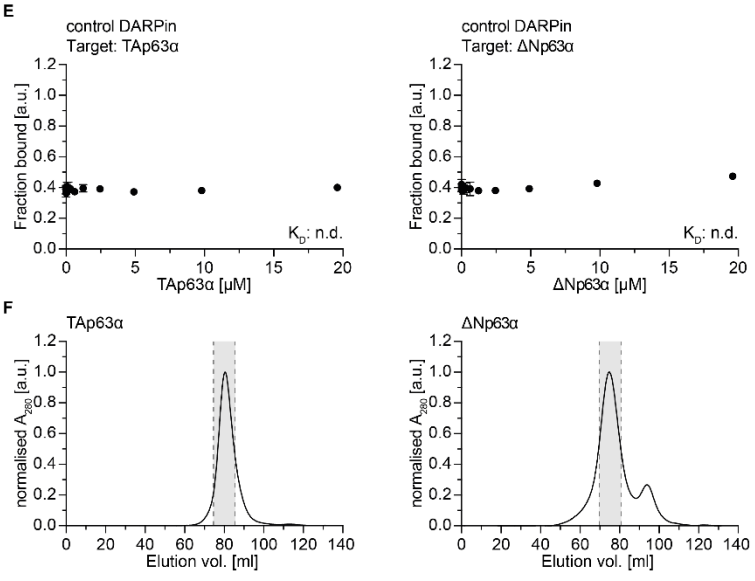
p63 DBD
time(s)
control DARPin
DP [µcal/s]
heat of injection [kcal/mol]
molar ratio
K_D: n.d.
C
p63 DBD + C14
p63 DBD
p63 DBD + G4
normalised A280 [a.u.]
Elution vol. [ml]
D
bait: cDP
prey: p53 family isoforms
Rel. PD efficiency [%]
TAp63α
ΔNp63α
TA*p63α
GTAp63α
TAp63γ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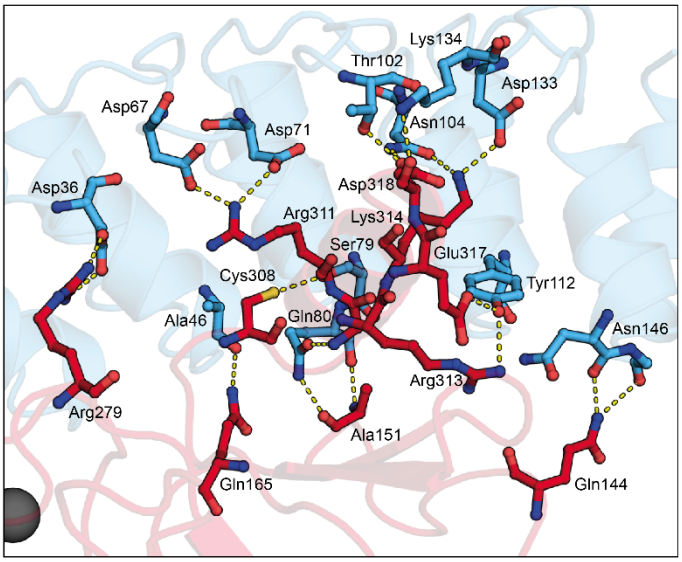
control DARPin
Target: TAp63α
Fraction bound [a.u.]
K_D: n.d.
TAp63α [µM]
control DARPin
Target: ΔNp63α
ΔNp63α [µM]
F
TAp63α
normalised A280 [a.u.]
Elution vol. [ml]
ΔNp63α

G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Asp133
Asp67
Asp71
Asn104
Asp36
Asp318
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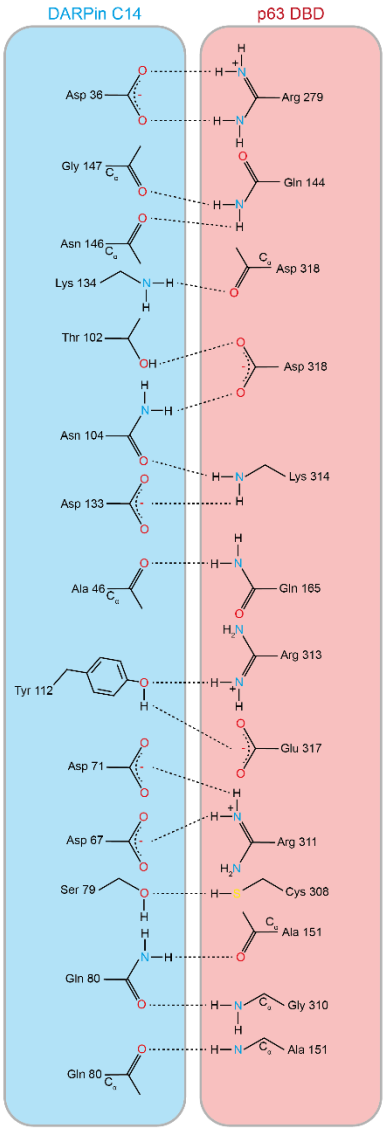
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p63 DBD
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Arg 279
Gly 147
Gln 144
Asn 146
Asp 318
Lys 134
Thr 102
Asp 318
Asn 104
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Asp 133
Ala 46
Gln 165
Arg 313
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Arg 311
Ser 79
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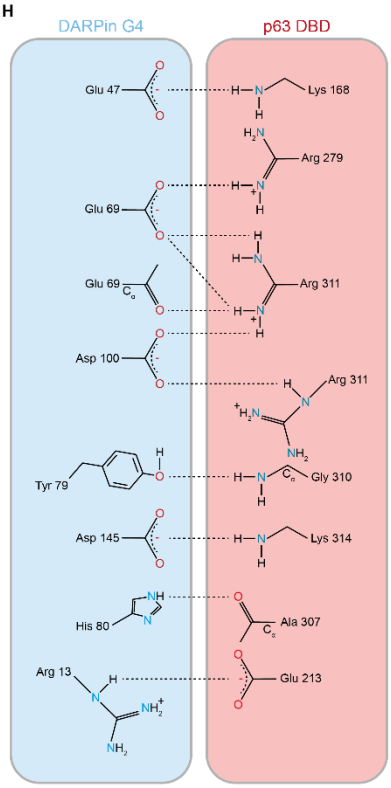
Ala 151
Gln 80
Gly 310
Ala 151
Gln 80

H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p63 DBD
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Lys 168
Arg 279
Glu 69
Glu 69
Arg 311
Asp 100
Arg 311
Tyr 79
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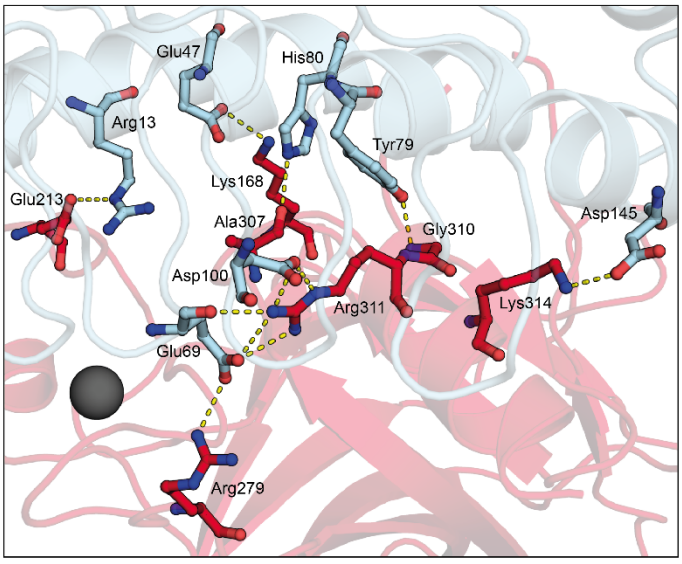
Lys 314
His 80
Ala 307
Arg 13
Glu 213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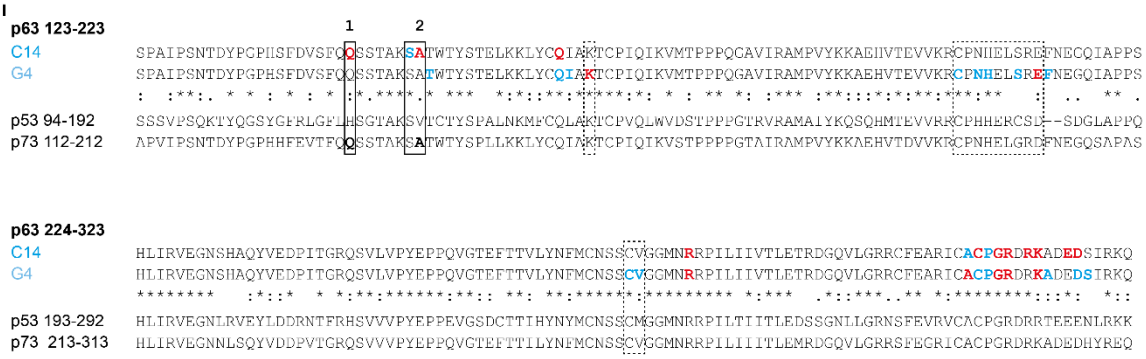
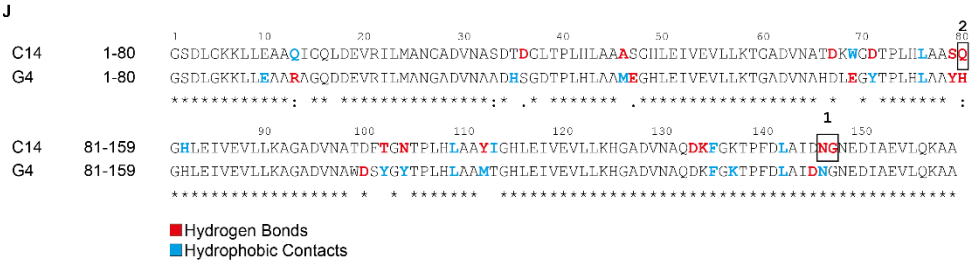
I


p63 123-223
C14
G4
p53 94-192
p73 112-212
1
2
Hydrogen Bonds
Hydrophobic Contacts
Unique interactions of DARPin C14
Unique interactions of DARPin G4

J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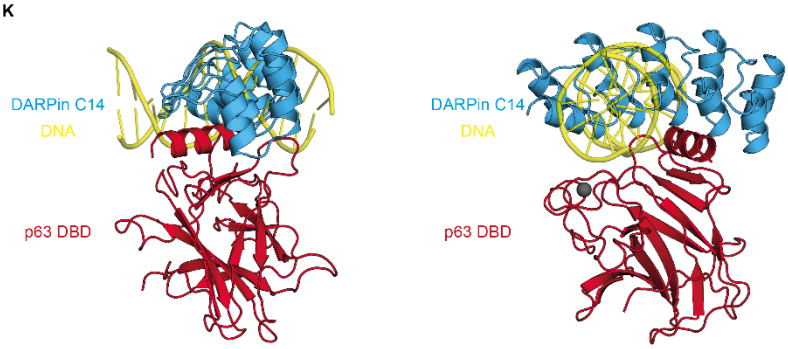
G4 1-80
C14 81-159
G4 81-159
1
2
Hydrogen Bonds
Hydrophobic Contacts

K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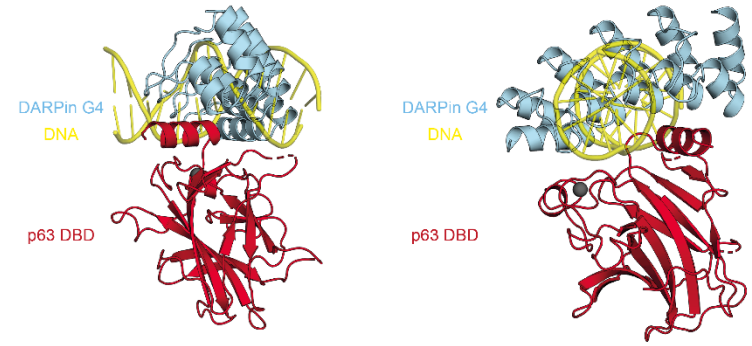
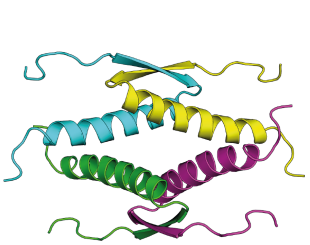
DNA
p63 DBD
DARPin C14
DNA
p63 DBD

L


DARPin G4
DNA
p63 DBD
DARPin G4
DNA
p63 DBD

A



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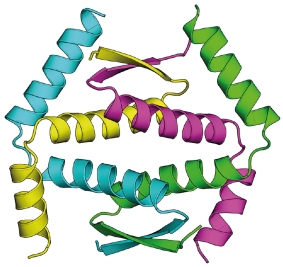
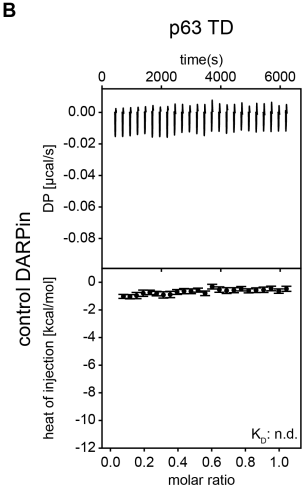


p63 TD

B



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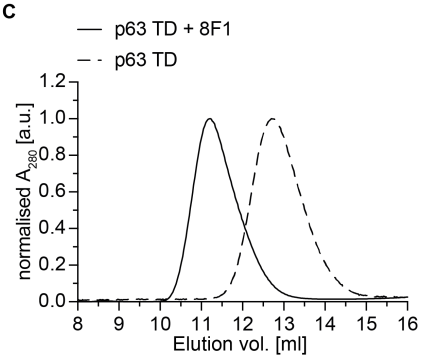

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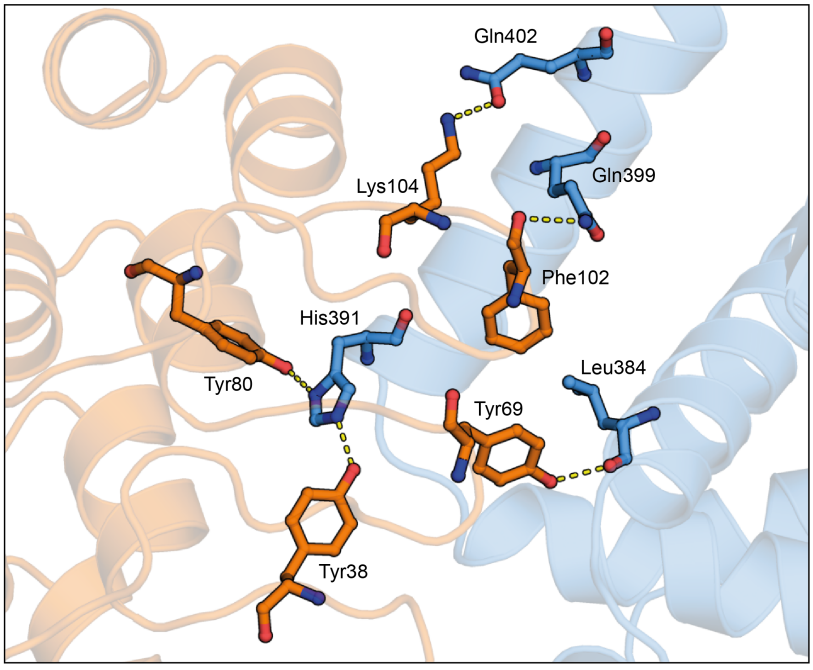

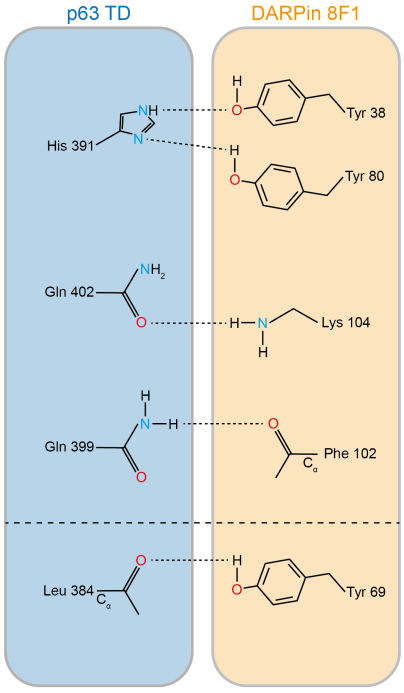

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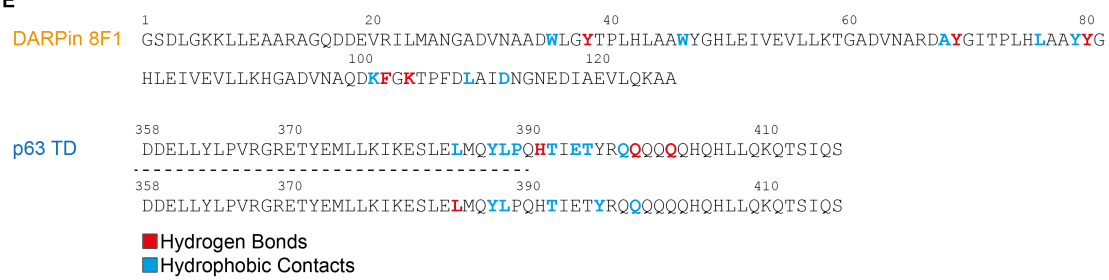

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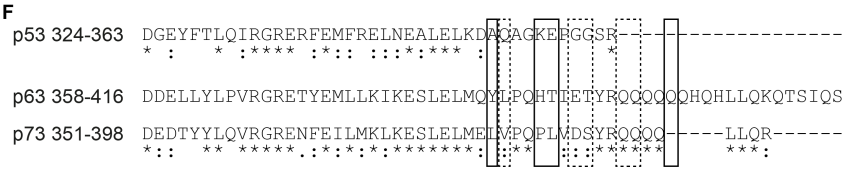

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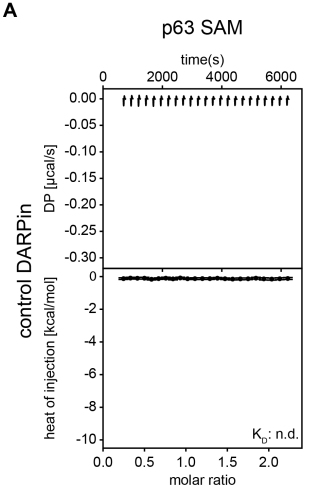
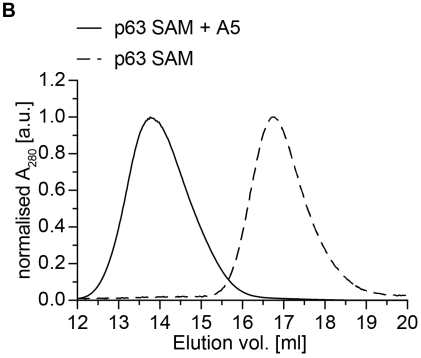
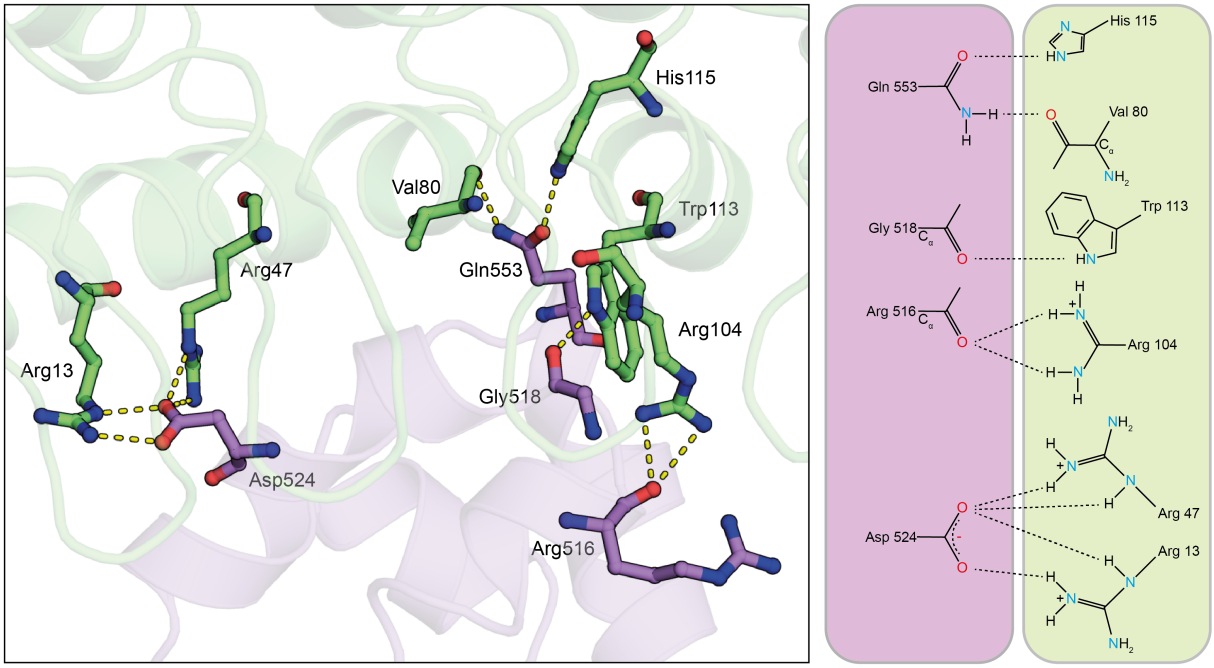

p63 SAM
time(s)
0
2000
4000
6000
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
DP [µcal/s]
control DARPin
0
-2
-4
-6
-8
-10
heat of injection [kcal/mol]
K_D: n.d.
0.0
0.5
1.0
1.5
2.0
molar ratio

B


p63 SAM + A5
p63 SAM
1.2
1.0
0.8
0.6
0.4
0.2
0.0
normalised A280 [a.u.]
12
13
14
15
16
17
18
19
20
Elution vol. [ml]

C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Trp113
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Gly518
Asp524
Arg516
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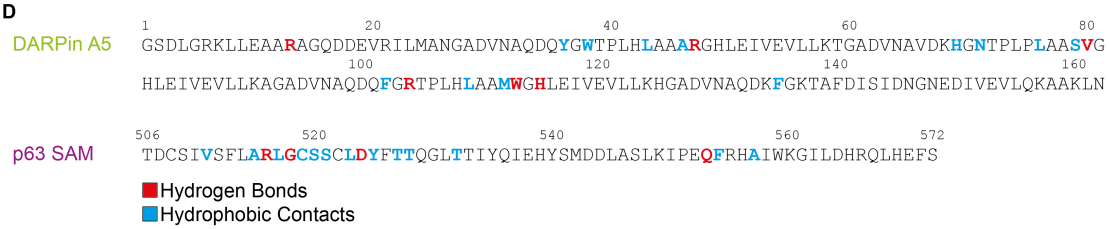
Val 80
Trp 113
Gly 518
Arg 516
Arg 104
Arg 47
Asp 524
Arg 13

D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20
40
60
80
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100
120
140
160
HLEIVEVLLKAGADVNAQDQFGRTPLHLAAMWGHLEIVEVLLKHGADVNAQDKFGKTAFDISIDNGNEDIVEVLQKAAKLN
p63 SAM
506
520
540
560
572
TDCSIVSFLARLGCSSCLDYFTTQGLTTIYQIEHYSMDDLASLKIPEQFRHAIWKGILDHRQLHEFS
Hydrogen Bonds
Hydrophobic Contacts

E

p63 506-572
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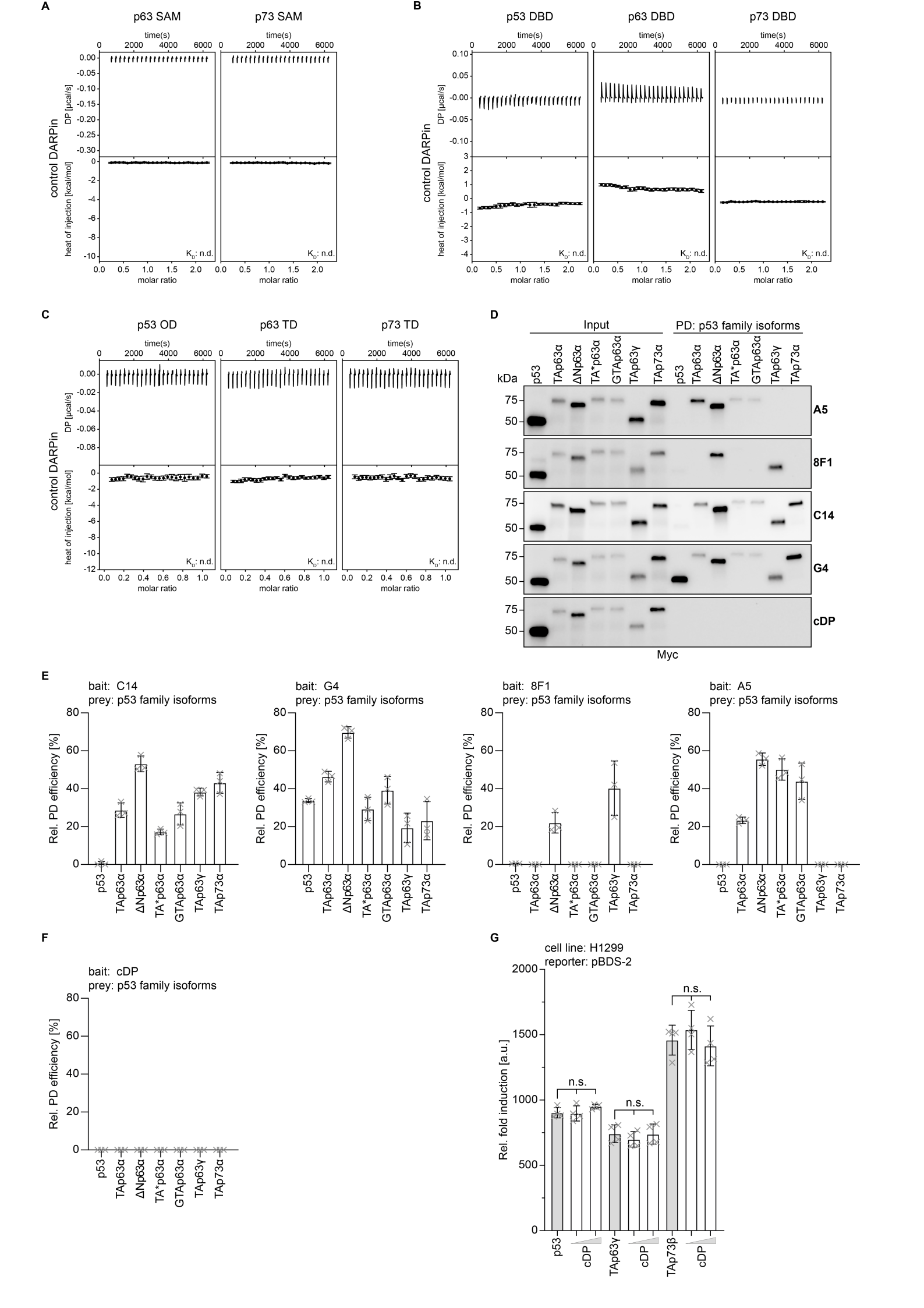
A
p63 SAM
p73 SAM
time(s)
control DARPin
DP [µcal/s]
heat of injection [kcal/mol]
molar ratio
K_D: n.d.
B
p53 DBD
p63 DBD
p73 DBD
C
p53 OD
p63 TD
p73 TD
D
Input
PD: p53 family isoforms
kDa
p53
TAp63α
ΔNp63α
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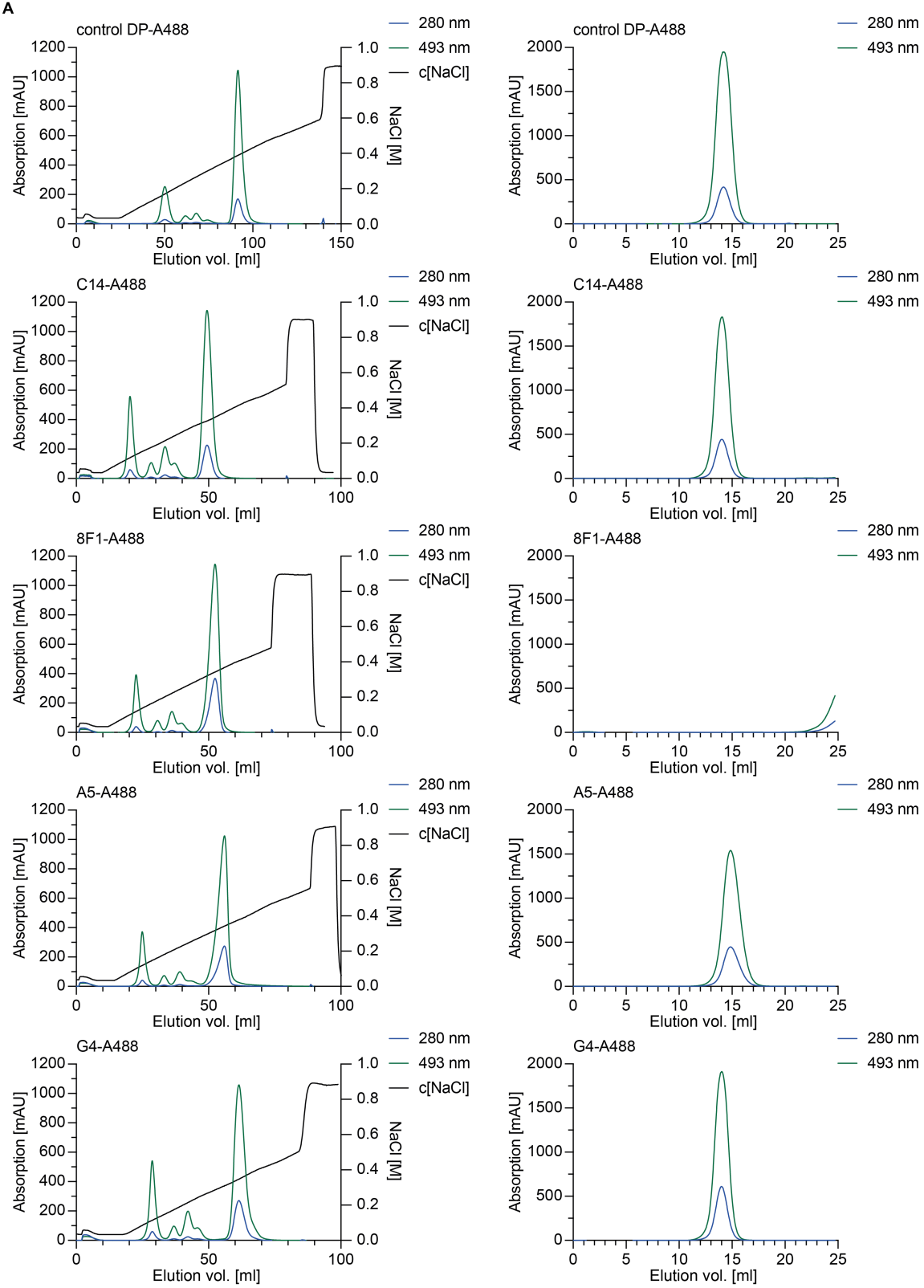
TAp63γ
TAp73α
A5
8F1
C14
G4
cDP
Myc
E
bait: C14
prey: p53 family isoforms
bait: G4
bait: 8F1
bait: A5
Rel. PD efficiency [%]
F
bait: cDP
G
cell line: H1299
reporter: pBDS-2
Rel. fold induction [a.u.]
n.s.
TAp63γ
TAp73β

A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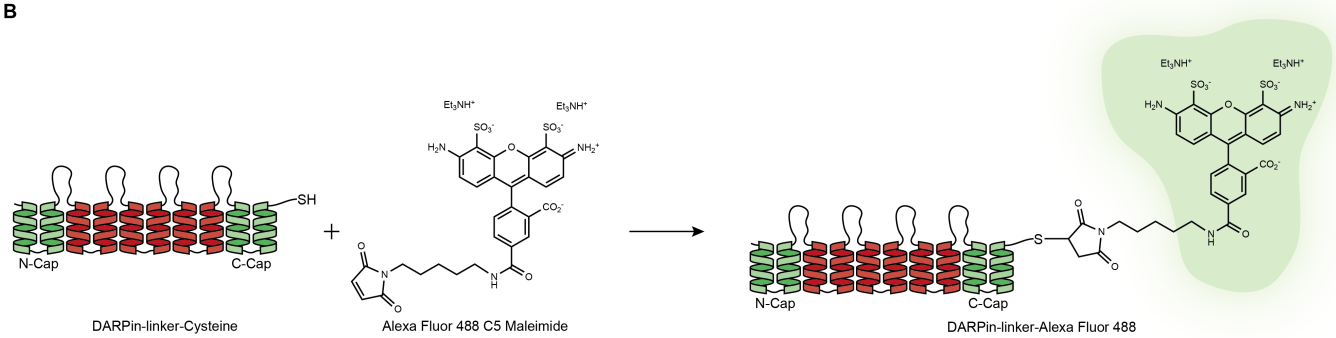
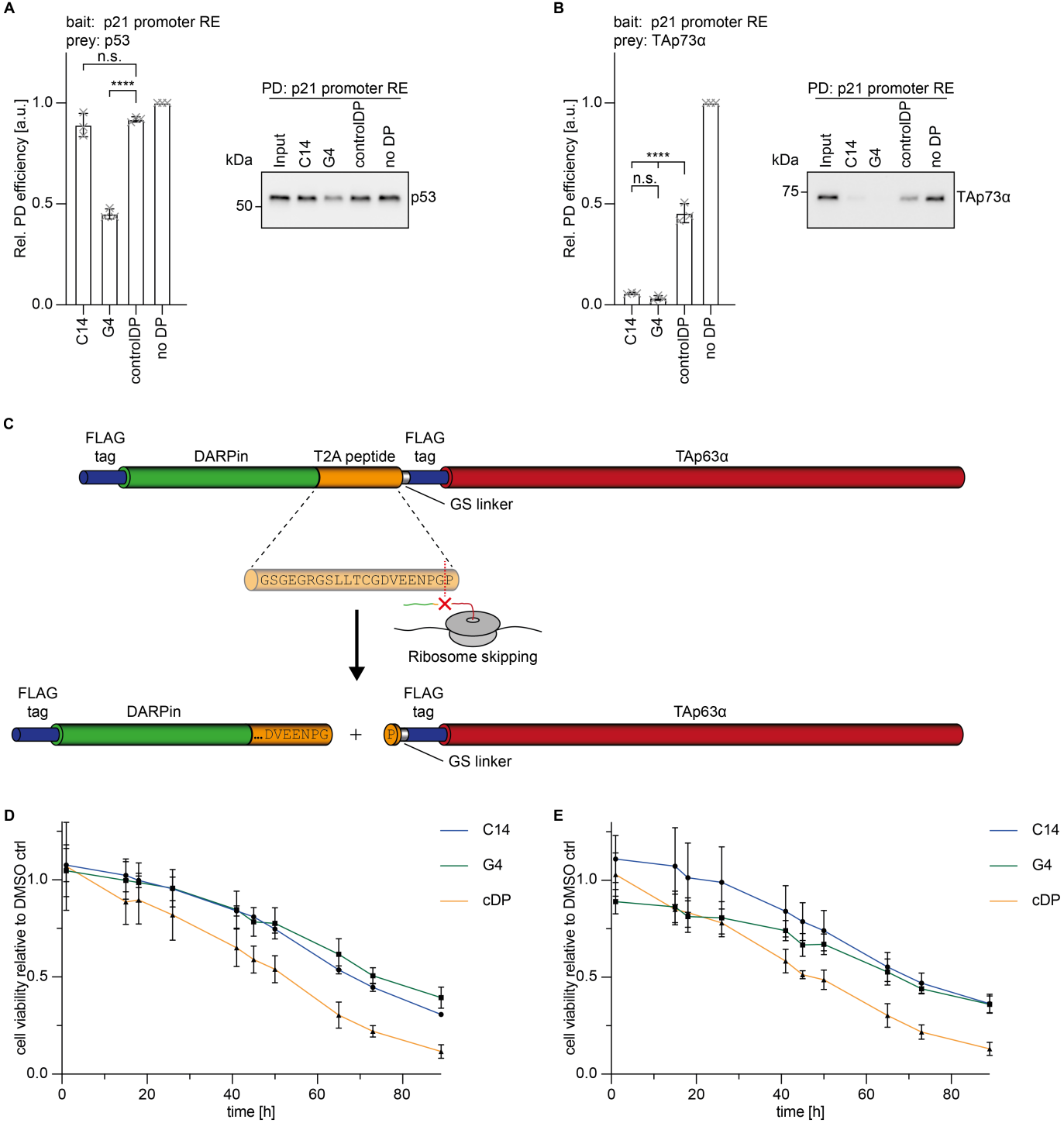
G4-A488
280 nm
493 nm
c[NaCl]
Absorption [mAU]
NaCl [M]
Elution vol. [ml]

B


SH
N-Cap
C-Cap
DARPin-linker-Cysteine
Alexa Fluor 488 C5 Maleimide
DARPin-linker-Alexa Fluor 488


A
bait: p21 promoter RE
prey: p53
n.s.
****
Rel. PD efficiency [a.u.]
1.0
0.5
0.0
C14
G4
controlDP
no DP
PD: p21 promoter RE
kDa
Input
C14
G4
controlDP
no DP
50
p53
B
bait: p21 promoter RE
prey: TAp73α
****
n.s.
Rel. PD efficiency [a.u.]
PD: p21 promoter RE
kDa
75
TAp73α
C
FLAG tag
DARPin
T2A peptide
FLAG tag
TAp63α
GS linker
GSGEGRGSLLTCGDVEENPGP
Ribosome skipping
FLAG tag
DARPin
...DVEENPG
+
P
FLAG tag
TAp63α
GS linker
D
cell viability relative to DMSO ctrl
1.0
0.5
0.0
0
20
40
60
80
time [h]
C14
G4
cDP
E
cell viability relative to DMSO ctrl
1.0
0.5
0.0
0
20
40
60
80
time [h]
C14
G4
cDP

DARPin	Target	T [°C]	K_A [$*10^6$ M^{-1}]	K_D [nM]	95% confidence interval min [nM]	95% confidence interval max [nM]	ΔH [kcal mol^{-1}]
A5	p63 SAM	25	61.80	16.18	11.48	22.39	-9.47
	p73 SAM	25	-	-	-	-	-
8F1	p53 OD	25	-	-	-	-	-
	p63 TD	25	18.61	53.73	41.04	69.86	-10.33
	p73 TD	25	-	-	-	-	-
C14	p53 DBD	25	-	-	-	-	-
	p63 DBD	10	9.30	107.50	74.30	151.88	1.88
	p73 DBD	25	62.74	15.94	10.08	23.38	-3.93
G4	p53 DBD	25	4.88	204.92	159.81	261.58	3.36
	p63 DBD	10	31.39	31.86	16.18	56.95	-3.72
	p73 DBD	25	4.48	223.11	180.14	274.79	2.95

95% confidence interval		ΔS	TΔS	ΔG
min [kcal mol^{-1}]	max [kcal mol^{-1}]	[cal mol^{-1} K^{-1}]	[kcal mol^{-1}]	[kcal mol^{-1}]
-9.68	-9.27	3.87	1.15	-10.63
-	-	-	-	-
-	-	-	-	-
-10.80	-9.90	-1.40	-0.42	-9.92
-	-	-	-	-
-	-	-	-	-
1.78	1.99	38.53	10.91	-9.03
-4.00	-3.85	22.51	6.71	-10.64
3.22	3.51	41.86	12.48	-9.13
-3.94	-3.51	21.82	6.51	-10.23
2.85	3.06	40.34	12.03	-9.07

DARPin	Target	T [°C]	K_D [nM]	95% confidence interval min [nM]	95% confidence interval max [nM]
A5	ΔNp63α	20	67.93	48.73	90.39
	TAp63α	20	296.50	190.90	437.30
8F1	ΔNp63α	20	95.61	73.49	120.90
	TAp63α	20	6159.00	4942.00	7691.00
C14	ΔNp63α	20	202.40	163.50	246.58
	TAp63α	20	733.90	514.00	1026.00
G4	ΔNp63α	20	137.20	118.70	157.40
	TAp63α	20	727.20	459.70	1111.00

Complex	DARPin C14-p63 DBD	DARPin A5-p63 SAM	DARPin 8F1-p63 TD	DARPin G4-p63 DBD
PDB accession code	**7Z71**	**7Z72**	**7Z73**	**7Z7E**
Data Collection				
Resolution[a] (Å)	44.64-1.85 (1.91-1.85)	45.92-1.80 (1.86-1.80)	47.06-2.27 (2.35-2.27)	48.29-1.80 (1.86-1.80)
Spacegroup	*P* 1	*P* 2_1	*P* 1	P 3_1 2 1
Cell dimensions	*a* = 53.0, *b* = 63.9, *c* = 65.5 Å *α* = 114.5°, *β* = 94.6°, *γ* = 104.1°	*a* = 48.5, *b* = 43.9, *c* = 50.0 Å *α, γ* = 90.0°, *β* = 113.3°	*a* = 42.6, *b* = 50.8, *c* = 65.9 Å *α* = 80.9°, *β* = 79.9°, *γ* = 68.8°	*A* = 96.6, *b* = 96.6, *c* = 77.1 Å *α,β* = 90.0°, *γ* = 120.0°
No. unique reflections[a]	58,153 (5,768)	17,870 (1,745)	21,523 (2,091)	37,301 (2,211)
Completeness[a] (%)	91.6 (92.6)	99.0 (99.6)	92.6 (91.5)	96.4 (96.6)
I/σI[a]	22.0 (9.5)	11.8 (2.0)	11.3 (2.2)	23.0 (1.9)
R_{merge}[a]	0.034 (0.110)	0.090(0.802)	0.055 (0.442)	0.019 (0.32)
CC (1/2)	0.998 (0.975)	0.998 (0.688)	0.997 (0.900)	0.99 (0.826)
Redundancy[a]	3.7 (3.8)	5.9 (6.0)	3.8 (3.9)	4.25 (1.07)
Refinement				
No. atoms in refinement (P/O)[b]	5,390/ 690	1,733/ 167	3,514/ 51	2,676/ 183
B factor (P/L/O)[b] ($Å^2$)	26/ 35	25/ 36	63/ 55	44/ 32/ 43
R_{fact} (%)	14.2	15.9	18.7	19.8
R_{free} (%)	18.1	18.8	23.5	24.7
rms deviation bond length[c] (Å)	0.016	0.014	0.011	0.010
rms deviation angle[c] (°)	1.5	1.4	1.2	1.6
Crystallization condition	25% PEG 3350, 0.2 M sodium chloride, 0.1 M HEPES pH 7.5	25% PEG3350, 0.1 M citrate pH 3.5	25% PEG3350, 0.2 M sodium chloride, 0.1 M bis-tris pH 5.5	25% PEG 3350, 0.2 M lithium sulfate, 0.1 M HEPES pH 7.5

[a] Values in brackets show the statistics for the highest resolution shells.

[b] P/O indicate protein and others (water and solvent molecules), respectively.

[c] rms indicates root-mean-square